New PCT Patent Application
Our Ref.: C 1748 PCT

Means and methods for modulating stomata characteristica in plants

The present invention relates to recombinant DNA molecules comprising nucleic acid molecules encoding subtilisin-like serine proteases that are involved in the regulation of stomatal density in plants; wherein said nucleic acid molecules could be operably linked to regulatory elements allowing the expression of the nucleic acid molecules in plants. The present invention also provides vectors comprising said recombinant DNA molecules as well as plant cells, plant tissues and plants transformed therewith. The present invention further relates to the use of the aforementioned recombinant DNA molecules and vectors in plant cell and tissue culture, plant breeding and/or agriculture, in particular for the production of plants with improved traits.

Several documents are cited throughout the text of this specification. Each of the documents cited herein (including any manufacturer's specifications, instructions, etc.) are hereby incorporated herein by reference; however, there is no admission that any document cited is indeed prior art as to the present invention.

Stomatal complexes (stomata) are specialised structures in the epidermices of all higher land plants that mediate and control the gas exchange between the internal tissues of the plants and the atmosphere. They consist of two guard cells that flank a central pore. In many plant species these central guard cells are surrounded by morphologically distinct epidermal cells (subsidiary cells). Usually, more than 90% of the gas exchange between a leaf and the atmosphere (uptake of CO_2 into the leaf and release of H_2O vapor) occurs through the stomatal pores. The major function of the stomata is to create an optimal balance between sufficient CO_2 uptake and limited water loss. To achieve this, short term control (in the range of minutes to hours) is exerted on the stomatal conductance by opening and closure of the stomatal pores through turgor driven movement of the guard cells (for review see Zeiger, Farquhar and Cowan (eds.) Stomatal Function,

Stanford University Press, Stanford 1987; Willmer and Fricker (eds.) Topics in Functional Biology, 2. Stomata, Second Edition, Chapman and Hall Ltd., London, New York, 1996). Besides these rapid and transient changes, long term modulation of stomatal characteristics occurs which predominantly involve morphological aspects such as presence or absence of stomata in the upper and/or lower leaf epidermis, density of stomata in the leaf epidermices, or the size of the stomata. These features are determined both, by endogenous (genetic) and by exogenous (environmental) factors. Hints towards genetic control were obtained through the observation of a broad variation of the stomatal density between different species of the same genus, between varieties or cultivars of the same species, or in F1 hybrids. Genetic analysis revealed multigenic, oligogenic, or monogenic control and a high heritability of characters such as stomatal density or size (for review see Jones, *In* Stomatal Function, Eds. E. Zeiger, G.D. Farquhar and I.R. Cowan, pp. 431-443, Stanford University Press, Stanford 1987). In addition to this endogenous control, stomatal characteristics are also modulated through environmental effects. Thus, air humidity (e.g. Schürmann, 1959, *Flora* 147, 417-520), light intensity (e.g. Gay and Hurd, 1975, *New Phytol.* 75, 37-46; Schoch et al., 1980, *J. Exp. Bot.* 31, 1211-1216; Rahim and Fordham, 1991, *Ann. Bot.* 67, 167-171), and CO_2-concentration (e.g. Woodward, 1987, *Nature* 327, 617-618; Woodward and Bazzaz, 1988, *J. Exp. Bot.* 39, 1771-1781; Goodfellow et al., 1997, *Tree Physiol.* 17, 291-299) were found to affect stomatal density. In several studies, stomatal density was found to be associated with plant yield (e.g. Walton, 1974, *Can J. Plant Sci.* 54, 749-754). Pima cotton varieties selected for high yield upon growth under conditions of high irradiance and artificial irrigation exhibit increased stomatal density associated with increased stomatal conductance and reduced leaf temperature (Cornish et al., 1991, *Plant Physiol.* 97, 484-489; Lu and Zeiger, 1994, *Physiol. Plant.* 92, 273-278; Lu et al., 1994, *Physiol. Plant.* 92, 266-272; Srivastava et al., 1995, *Plant Sci.* 19, 125-131). A similar relationship between stomatal conductance and yield was observed for a series of bread wheat varieties (Lu et al., 1998, *J. Exp. Bot.* 49, 453-460). According to these data, the modulation of stomatal characteristics are of high importance for the improvement of elite cultivars of crop plants. In the area of agriculture and forestry, a major aim is the continuous improvement of the crop plants with respect to higher yielding to provide sufficient food for the growing

global population and to ensure the supply of renewable resources. Traditionally, progress towards higher yielding varieties is attempted through breeding, a very labour und time consuming process to be conducted separately for every relevant plant species. Some progress has already been achieved through the application of genetic engineering to plants, i.e. the introduction and expression of recombinant nucleic acid molecules in plants. Such approaches are advantageous as they can usually be applied to many different plant species. In EP-A 0 511 979, for instance, the use of a procaryotic asparagine synthetase for expression in plant cells is described that, among other changes, leads to increased biomass production. WO 96/21737 describes yield increases in plants achieved through the expression of de- or non-regulated fructose-1,6-bisphosphatase through enhanced rate of photosynthesis. In WO 96/17069, the enhancement of biomass production in transgenic plants achieved through expression of a polyphosphate kinase from *E.coli* is described. In contrast to these cases, however, no means for a directed manipulation through genetic engineering of stomatal density or distribution in plants were hitherto available, due to the complete lack of knowlegde about genes that are involved in the control of these stomatal characteristics.

Recently, an *Arabidopsis thaliana* mutant, R-558, has been isolated after chemical mutagenesis which shows a two to four-fold increase in the stomatal density of all aerial plant organs, in the leaves in particular and the occurrence of ca. 10% clustered stomata, i.e. stomata placed in direct contact to at least one other stomata (D. Berger, 1997, PhD Thesis Freie Universität Berlin). Besides a minor change in the length of the pedicelli, no other morphological changes were visible in the mutant plants. The form and size of the leaves as well as the structure of the mesophyll (number of cell layers in palisade and spongy parenchyma, form and size of the mesophyll cells) and the intercellular system (including the substomatal cavities) are unchanged. The increased stomatal density resulted in elevated transpiration (loss of H_2O) and was associated with increased dry matter content in the leaves which in the wild type was ca. 3% and in the mutant ca. 7%. It was furthermore shown that the increased stomatal density in the R-558 mutant was associated with increased leaf fresh (+ 15%) and dry (+30%) weight, increasd glucose (+70%), fructose (+65%), and protein (+50%) contents in leaves, and enhanced transpiration and CO_2-assimilation (D. Berger, 1997, PhD Thesis, Freie

Universität Berlin) in comparison to the wild type. The mutation which caused the increased stomatal density has been mapped relative to a set of (molecular) genetic markers to a ca. 0.59 cM interval located on the top arm of chromosome 1 of *Arabidopsis thaliana* (D. Berger, 1997, PhD Thesis, Freie Universität Berlin). However, the regulation of stomatal density and distribution in plants is still not fully understood and means that can be used to manipulate stomatal characteristics such as density and distribution that may have applications in several aspects of agriculture were hitherto not available.

Thus, the technical problem underlying the present invention was to comply with the need for means and methods for modulating the stomatal density in plants. The solution to this technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the invention relates to a recombinant DNA molecule comprising

(i) a nucleic acid molecule encoding a subtilisin-like serine protease or encoding a biologically active fragment of such a protein, selected from the group consisting of

(a) nucleic acid molecules comprising a nucleotide sequence encoding a protein comprising the amino acid sequence as given in SEQ ID NO: 2, 8, 10 or 12;

(b) nucleic acid molecules comprising a nucleotide sequence as given in SEQ ID NO: 1, 7, 9, or 11;

(c) nucleic acid molecules encoding a protein comprising at least the D region, H region, substrate binding site and/or S region of the subtilisin-like serine protease encoded by a nucleic acid molecule of (a) or (b); or

(d) nucleic acid molecules hybridizing with the complementary strand of a nucleic acid molecule as defined in any one of (a) to (c);

(e) nucleic acid molecules encoding a protein the amino acid sequence of which is at least 65% identical to the amino acid sequence encoded by a nucleic acid molecule of any one of (a) to (c);

(f) nucleic acid molecules, the nucleotide sequence of which is degenerate as a result of the genetic code to a nucleotide sequence of a nucleic acid molecule as defined in any one of (a) to (e); or

(ii) a nucleic acid molecule encoding a mutant non-active or hyper-active form of or an antibody against the subtilisin-like serine protease encoded by a nucleic acid molecule of (i); or

(iii) a nucleic acid molecule which specifically hybridizes with a nucleic acid molecule of (i) or the complementary strand thereof.

The present invention is based on the identification of a new class of genes represented by *SDD1* from *Arabidopsis thaliana* which share common structural motifs, see infra. In one aspect, these genes are preferably involved in the control of stomatal density and/or distribution. The SDD1 gene is mutated in the *Arabidopsis thaliana* mutant R-558; see Examples 1 to 3. Computer-assisted amino acid sequence analysis of the protein encoded by this gene revealed that it belongs to a family of subtilisin-like serine proteases; see Example 4. Further representatives of this new class of genes have been cloned from potato; see Example 6.

The terms "subtilisin like-serine protease" and "subtilase" are used interchangeable herein and mean a specific class of serine proteases, called subtilisins or dibasic processing endoproteases. In subtilisins, four regions form the catalytic triad and the substrate binding site and are most highly conserved among subtilisins; see also Example 4 and Figure 7. In the context of the present invention, subtilisin-like serine proteases also mean such proteins which show a homology of at least 65% to the sequence shown in SEQ ID NOs: 2, 8, 10 or 12. In the context of the present invention, the term "subtilisin-like serin protease" preferably is understood to mean proteins comprising one or several of the characteristic motifs depicted in SEQ ID NOS: 13 to 37; see infra.
The substrate binding site preferably comprises the motif VICAA (SEQ ID NO: 38), more preferably the motif CAAGN (SEQ ID NO: 39), in particular the motif AAGNN (SEQ ID NO: 40) and most preferably the amino acid motif VICAAGNNG (SEQ ID NO: 41).

In another preferred embodiment, the nucleic acid molecule of the present invention encodes a protein described above with one or more of the following amino acid sequence motifs: SYHSA (SEQ ID NO: 49), GLSPT (SEQ ID NO: 50), WLKSG (SEQ ID NO: 51), FNSSS (SEQ ID NO: 52), ASTAG (SEQ ID NO: 53), AAMDV (SEQ ID NO: 54), WIATI (SEQ ID NO: 55), GPSGL (SEQ ID NO: 56), IAALLH (SEQ ID NO: 57), KPIMD (SEQ ID NO: 58), VSCHD (SEQ ID NO: 59), YPSIS (SEQ ID NO: 60), SLSYR (SEQ ID NO: 61).

In a further preferred embodiment the D, H and/or S region of the subtilase of the present invention comprise one or several of the following characteristic motifs:

D region:

IIGVL (SEQ ID NO: 42) or GVLDT (SEQ ID NO: 43)

H region:

THTAST (SEQ ID NO: 44) or S-RDS (SEQ ID NO: 45) or RDS-G (SEQ ID NO:46)

S region:

HVSGI (SEQ ID NO: 47) or FTV-SGT (SEQ ID NO: 48)

While a function of such proteins in the regulation of stomatal density in plants was hitherto unknown, the present invention for the first time provides evidence that the described nucleic acid molecules encode proteins that are involved in controlling the density and the distribution of stomata in plants; see Examples 3 and 7. Furthermore, it is shown that plants lacking or overexpressing such proteins show altered morphological and physiological features of high agronomic importance.

Thus, the present invention for the first time clearly establishes that stomatal characteristics such as density and distribution can be specifically modulated through the application of genetic engineering techniques and provides extremely useful tools for example to:

(i) generate plants with increased stomatal density and consequently with enhanced CO_2 assimilation, reduced leaf temperature, enhanced organ such as leaf fresh and dry weight, and enhanced sugar and protein contents in organs such as leaves;

(ii) generate plants with decreased stomatal density and consequently with reduced water loss and thus lower water consumption;

(iii) counteract environmental changes such as raises in atmospheric CO_2 levels, temperature and irradiation that would cause changes in stomatal density to sub- or supra-optimal levels;

In general a nucleic acid molecule encoding a subtilisin-like serine protease can be derived from any material source, for example, from any organism, preferably plants possessing such molecules, preferably form monocotyledonous or dicotyledonous plants, in particular from any plant of interest in agriculture, horticulture or wood culture, such as crop plants, namely those of the family Poaceae, any starch producing plants, such as potato, maniok, rice, wheat, corn, barley, oat, leguminous plants, oil producing plants, such as oilseed rape, soja, sunflower, linenseed, etc., plants using polypeptide as storage substances, such as soybean, plants using sucrose as storage substance, such as sugar beet or sugar cane, trees, ornamental plants etc. or plants belonging to the family Gramineae.

Furthermore, nucleic acid molecules can be used in accordance with the present invention hybridizing to the above-described nucleic acid molecules and encoding subtilisin-like serine protease. Such nucleic acid molecules can be isolated, e.g., from libraries, such as cDNA or genomic libraries by techniques well known in the art. For example, hybridizing nucleic acid molecules can be identified and isolated by using the above-described nucleic acid molecules or fragments thereof or complements thereof as probes to screen libraries by hybridizing with said molecules according to standard techniques. Possible is also the isolation of such nucleic acid molecules by applying the polymerase chain reaction (PCR) using as primers oligonucleotides derived form the above-described nucleic acid molecules. Nucleic acid molecules which hybridize with any of the aforementioned nucleic acid molecules also include fragments, derivatives and allelic variants of the above-described nucleic acid molecules that encode subtilisin-like serine proteases or biologically active fragments thereof. Fragments are understood to be parts of nucleic acid molecules long enough to encode the described protein or a fragment thereof having the biological activity as defined above. Preferably, said fragment comprises at least one region of subtilisin-like serine protease as defined in section (i) (c) supra.

The term "derivative" means in this context that the nucleotide sequence of these nucleic acid molecules differs from the sequences of the above-described nucleic acid molecules in one or more nucleotide positions and are highly homologous to said nucleic acid molecules. Homology is understood to refer to a sequence identity of at least 50 %, preferably 65% identity, particularly an identity of at least 70 % or 75%, preferably more than 80 % and still more preferably more than 90 % or 95% identity. The deviations from the sequences of the nucleic acid molecules described above can, for example, be the result of nucleotide substitution(s), deletion(s), addition(s), insertion(s) and/or recombination(s) either alone or in combination, that may naturally occur or be produced via recombinant DNA techniques well known in the art; see for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989), (1994). Homology further means that the respective nucleic acid molecules or encoded proteins are functionally and/or structurally equivalent. The nucleic acid molecules that are homologous to the nucleic acid molecules described above and that are derivatives of said nucleic acid molecules are, for example, variations of said nucleic acid molecules which represent modifications having the same biological function, in particular encoding proteins with the same or substantially the same biological activity as defined herein. They may be naturally occurring variations, such as subtilisin-like serine protease encoding sequences from other prokaryotes and eukaryotes, respectively, or mutations. These mutations may occur naturally or may be obtained by mutagenesis techniques; see supra. The allelic variations may be naturally occurring allelic variants as well as synthetically produced or genetically engineered variants; see supra. For example, the amino acid sequences of plant subtilisin-like serine proteases share significant similarities with bacterial, yeast and mammalian subtilisin-like serine protease; see Example 4. In addition, nucleic acid molecules can be employed in accordance with the present invention that encode homologs or analogs of the above described subtilisin-like serine protease but where otherwise unrelated to those proteins. Such proteins that do not display significant homologies to common subtilisin-like serine protease can be identified by a person skilled in the art using techniques

well known in the art, for example, via complementation of mutant genes, for example, in corresponding mutant plants; see Example 3.

In a further embodiment the term derivative encompasses a nucleotide sequence coding for a protein derived from any one of SEQ ID Nos. 2, 8, 10 or 12 which exhibits a degree of homology, i.e. identity to the protein depicted under SEQ ID Nos. 2, 8, 10 or 12 of at least 60%, in particular an homology of at least 70%, preferably more than 80% and still more preferably a homology of more than 90% and particularly preferred of more than 95% and which exhibits at least one, more preferably at least three, even more preferably of at least five, in particular at least ten and particularly preferred of at least twenty of the peptide motifs selected from the group consisting of

a) QTYIV, (SEQ ID NO: 13),
b) IVQLH, (SEQ ID NO: 14),
c) SSRLL, (SEQ ID NO: 15),
d) QTTYS, (SEQ ID NO: 16),
e) SSSCN, (SEQ ID NO: 17),
f) VLGNG, (SEQ ID NO: 18),
g) GAHIA, (SEQ ID NO: 19),
h) FRAME, (SEQ ID NO: 20),
i) VICAA, (SEQ ID NO: 21),
j) AAGNN, (SEQ ID NO: 22),
k) SSVAN, (SEQ ID NO: 23),
l) YGESL, (SEQ ID NO: 24),
m) GSEFC, (SEQ ID NO: 25),
n) CLRGS, (SEQ ID NO: 26),
o) RGVNG, (SEQ ID NO: 27),
p) PATLIG, (SEQ ID NO: 28),
q) IFGGT, (SEQ ID NO: 29),
r) PQNLG, (SEQ ID NO: 30),
s) VNFTV, (SEQ ID NO: 31),
t) HVSGI, (SEQ ID NO: 32),
u) GFSLN, (SEQ ID NO: 33),
v) RRVTN, (SEQ ID NO: 34),
w) PNSIY, (SEQ ID NO: 35),
x) LSYRV, (SEQ ID NO: 36), and
y) SPISV. (SEQ ID NO: 37)

The proteins encoded by the various derivatives, variants, homologs or analogs of the above-described nucleic acid molecules may share specific common characteristics, such as molecular weight, immunological reactivity, conformation, etc., as well as physical properties, such as electrophoretic mobility, chromatographic behavior, sedimentation coefficients, pH optimum, temperature

optimum, stability, solubility, spectroscopic properties, etc. All these nucleic acid molecules and derivatives can be employed in accordance with the present invention as long as the biological activity of the encoded protein remains substantially unaffected in kind, namely that the protein is capable of modulating stomata density in plants. Any one of the above described nucleic acid molecules, in particular those that represent hyper-active mutant forms of subtilisin-like serine proteases are particular suitable for overexpression in transgenic plants. These transgenic plants may either possess an endogenous functional subtilisin-like serine protease or they may lack the corresponding genes, e.g. due to mutation.

The nucleic acid molecules mentioned in section (ii) and (iii) are particular useful for the suppression of genes encoding subtilisin-like serine proteases in plants. Hence, in one embodiment said nucleic acid molecules are preferably of at least 50 nucleotides in length hybridizing specifically with a nucleic acid molecule as described above or with a complementary strand thereof. Specific hybridization occurs preferably under stringent conditions and implies no or very little cross-hybridization with nucleotide sequences encoding no or substantially different proteins. In particular stringent conditions mean, e.g., the use of an aqueous solution of 1% BSA, 1mM EDTA, 0.5 M $NaHPO_4$ pH7.2, 7% SDS and incubation at 65°C. Preferably, stringent hybridization is obtained under the following conditions:

Hybridization buffer:

2 x SSC; 10 x Denhardt's solution (Ficoll 400 + PEG + BSA; ratio 1:1:1); 0.1% SDS; 5mM EDTA; 50 mM Na_2HPO_4; 250 µg/ml herring sperm DNA; 50 µ/ml tRNA; or

0.25M sodium phosphate buffer pH 7.2; 1mM EDTA; 7% SDS

Hybridization temperature:	T=65 to 68°C
Washing buffer:	0.2 x SSC; 0.1% SDS
Washing temperature:	T=68°C

Such nucleic acid molecules may be used as probes and/or for the control of gene expression. Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary in length. Preferred are nucleic acid probes of 50 nucleotides or more in length. Of course, it may also be

appropriate to use nucleic acids of up to 100 and more nucleotides in length. The nucleic acid probes of the invention are useful for various applications. On the one hand, they may be used as PCR primers for amplification of nucleic acid sequences according to the invention. Another application is the use as a hybridization probe to identify nucleic acid molecules hybridizing with a nucleic acid molecule of the invention by homology screening of genomic DNA or cDNA libraries. Nucleic acid molecules according to this preferred embodiment of the invention which are complementary to a nucleic acid molecule as described above can be used for repression of expression of a subtilisin-like serine protease encoding gene, for example due to an antisense or triple helix effect or for the construction of appropriate ribozymes (see, e.g., EP-A1 0 291 533, EP-A1 0 321 201, EP-A2 0 360 257) which specifically cleave the (pre)-mRNA of a gene comprising a nucleic acid molecule of the invention or part thereof. Selection of appropriate target sites and corresponding ribozymes can be done as described, for example, in Steinecke, Ribozymes, Methods in Cell Biology 50, Galbraith et al. eds Academic Press, Inc. (1995), 449-460. Furthermore, the person skilled in the art is well aware that it is also possible to label such a nucleic acid probe with an appropriate marker for specific applications, such as for the detection of the presence of a nucleic acid molecule of the invention in a sample derived from an organism, in particular plants.

The above described nucleic acid molecules may either be DNA or RNA or a hybrid thereof. Furthermore, said nucleic acid molecule may contain, for example, thioester bonds and/or nucleotide analogues, commonly used in oligonucleotide anti-sense approaches. Said modifications may be useful for the stabilization of the nucleic acid molecule against endo- and/or exonucleases in the cell.

Furthermore, nucleic acid molecules encoding antibodies specifically recognizing a subtilisin-like serine protease or parts, i.e. specific fragments or epitopes, of such a protein can be used for inhibiting the activity of the protein in plants. These antibodies can be monoclonal antibodies, polyclonal antibodies or synthetic antibodies as well as fragments of antibodies, such as Fab, Fv or scFv fragments etc. Monoclonal antibodies can be prepared, for example, by the techniques as originally described in Köhler and Milstein, Nature 256 (1975), 495, and Galfré, Meth. Enzymol. 73 (1981), 3, which comprise the fusion of mouse myeloma cells to

spleen cells derived from immunized mammals. Furthermore, antibodies or fragments thereof to the aforementioned peptides can be obtained by using methods which are described, e.g., in Harlow and Lane "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbor, 1988. These antibodies can be used, for example, for the immunoprecipitation and immunolocalization of proteins according to the invention as well as for the monitoring of the synthesis of such proteins, for example, in recombinant organisms, and for the identification of compounds interacting with the protein according to the invention. For example, surface plasmon resonance as employed in the BIAcore system can be used to increase the efficiency of phage antibodies selections, yielding a high increment of affinity from a single library of phage antibodies which bind to an epitope of the protein of the invention (Schier, Human Antibodies Hybridomas 7 (1996), 97-105; Malmborg, J. Immunol. Methods 183 (1995), 7-13). Expression of antibodies or antibody-like molecules in plants can be achieved by methods well known in the art, for example, full-size antibodies (Düring, Plant. Mol. Biol. 15 (1990), 281-293; Hiatt, Nature 342 (1989), 469-470; Voss, Mol. Breeding 1 (1995), 39-50), Fab-fragments (De Neve, Transgenic Res. 2 (1993), 227-237), scFvs (Owen, Bio/Technology 10 (1992), 790-794; Zimmermann, Mol. Breeding 4 (1998), 369-379; Tavladoraki, Nature 366 (1993), 469-472) and dAbs (Benvenuto, Plant Mol. Biol. 17 (1991), 865-874) have been successfully expressed in Tobacco, Potato (Schouten, FEBS Lett. 415 (1997), 235-241) or *Arabidopsis*, reaching expression levels as high as 6.8% of the total protein (Fiedler, Immunotechnology 3 (1997), 205-216).

In addition, nucleic acid molecules encoding mutant forms of a subtilisin-like serine protease can be used to interfere with the activity of the wild type protein. Such mutant forms preferably have lost their biological activity as defined above and may be derived from the corresponding subtilisin-like serine protease by way of amino acid deletion(s), substitution(s), and/or additions in the amino acid sequence of the protein. As mentioned above, mutant forms of subtilisin-like serine proteases also encompass hyper-active mutant forms of such proteins which display, e.g. an increased substrate affinity and/or higher substrate turnover of the same. Furthermore, such hyper-active forms may be more stable in the cell due to the incorporation of amino acids that stabilize proteins in the cellular environment.

These mutant forms may be naturally occurring or genetically engineered mutants; see also supra.

The recombinant DNA molecule of the invention preferably comprises regulatory sequences allowing for the expression the nucleic acid molecules in plants. Preferably, said regulatory elements comprise a promoter active in plant cells. Expression comprises transcription of the nucleic acid molecule preferably into a translatable mRNA. Regulatory elements ensuring expression in plant cells are well known to those skilled in the art.

These regulatory elements may be homologous or preferably heterologous with respect to the nucleic acid molecule to be expressed and/or with respect to the plant species to be transformed. For example, a preferred regulatory element that can be employed in accordance with the present invention is the SDD1 promoter region as depicted in SEQ ID NO: 5 or part thereof.

Preferably, the promoter region of the SDD1 gene comprising SEQ ID NO: 6 is employed, which corresponds to the nucleotide sequence of SEQ ID NO: 5 starting at position 839. GUS expression studies show that the promoter of SDD1 of Arabidopsis thaliana in tissues having mitotic activity shows a high activity. For example, a very strong GUS expression can be found in developing stomata and in primordials, but also a weaker expression in lateral roots. By way of computer-assisted studies different domains could be identified which possibly are responsible for the expression pattern of this promoter. On the one hand, a domain was identified which allows expression in roots, on the other hand several characteristic motifs were identified which are termed Dof-motifs (see, e.g., Yanagisawa and Schmidt, Plant J. 17 (1999), 209-214) and which in the present case possibly allow for an expression in guard cells. These motifs have, e.g., been described in German patent application DE 19904754.5. It is assumed that a deletion of the domain, which possibly mediates the expression in roots and which is located within the first 400bp of SEQ ID NO: 6, advantageously changes the specificity of the promoter. For this reason preferred embodiments of the invention use promoter fragments comprising at the 5'-region a deletion of at least 400-450bp or of 450-600bp or at the most 900bp.

It is possible for the person skilled in the art to isolate with the help of the coding and regulatory sequences of the invention corresponding genes from other species, for example, potato, tomato, barley, wheat, oat, rye, rice, corn, soja, etc. This can be done by conventional techniques known in the art, for example, by using the regulatory sequences depicted in SEQ ID NO: 6 as a hybridization probe or by designing appropriate PCR primers. It is then possible to isolate the corresponding promoter region by conventional techniques and test it for its expression pattern. For this purpose, it is, for instance, possible to fuse the promoter to a reporter gene, such as GUS, luciferase or green fluorescent protein (GFP) and assess the expression of the reporter gene in transgenic plants.

For example the promoters from the two SDD1 homologs from Solanum toberosum described in Example 6 can be isolated by conventional means. Genomic clones can be amplified, e.g., fragments via long template PCR (employing for example the EXPAND kit, Boehringer Mannheim), using an upstream oriented SDD1 specific primer and a primer to the Lambda left or right arm sequence. The amplified fragment is sequenced via primer walking until several kb upstream from the transcription start point have been reached, if present on the clone, preferably more than 3 kb. Within the cloned genomic sequence, the transcription start site is determined by standard procedures well known to the person skilled in the art, such as 5'-RACE, primer extension or S1 mapping. To define cis-regulatory elements upstream of the transcription start site (i.e. within the putative promoter region), the respective region is fused to marker genes such as genes encoding GUS or GFP, and 5' deletion derivatives of these construct are generated. They are transformed into suitable plant material, and the expression of the marker gene depending on the remaining upstream sequence (putative promoter) is determined. These techniques are well known to the person skilled in the art.

The regulatory sequences so identified may differ at one or more positions from the above-mentioned regulatory sequence but still have the same specificity, namely they comprise the same or similar sequence motifs, preferably 6 to 10 nucleotides in length, responsible for the above described expression pattern. Preferably such regulatory sequences hybridize to one of the above-mentioned regulatory sequences, most preferably under stringent conditions. Particularly

preferred are regulatory sequences which share at least 85%, more preferably 90-95%, and most preferably 96-99% sequence identity with one of the above-mentioned regulatory sequences and have the same or substantially the same specificity. Particularly preferred are the regulatory sequences that comprise the above mentioned motifs which allow for an expression in guard cells. Such regulatory sequences also comprise those which are altered, for example by nucleotide deletion(s), insertion(s), substitution(s), addition(s), and/or recombination(s) and/or any other modification(s) known in the art either alone or in combination in comparison to the above-described nucleotide sequence. Methods for introducing such modifications in the nucleotide sequence of the regulatory sequences of the invention are well known to the person skilled in the art. It is also immediately evident to the person skilled in the art that further regulatory elements may be added to the regulatory sequences of the invention. For example, transcriptional enhancers and/or sequences which allow for induced expression of the regulatory sequences of the inverition may be employed.

The possibility exists to modify the regulatory sequences as described above or sequence motifs thereof by, e.g., nucleotide replacements which do not affect the overall structure or binding motif of the regulatory sequence so that it remains capable of conferring the gene expression pattern as described above. Such regulatory sequences may be derived from subtilase genes of potato although other plants may be suitable sources for such regulatory sequences as well. Furthermore, the nucleotide sequences can be compared using appropriate computer programs known in the art such as BLAST, which stands for Basic Local Alignment Search Tool (Altschul, 1997; Altschul, J. Mol. Evol. 36 (1993), 290-390; Altschul, J. Mol. Biol. 215 (1990); 403-410), to search for local sequence alignments. BLAST produces alignments of nucleotide sequences to determine sequence similarity. Because of the local nature of the alignments, BLAST is especially useful in determining exact matches or in identifying homologues.

In general, regulatory elements employed in accordance with the present invention comprise a promoter active in plant cells. To obtain expression in all tissues of a transgenic plant, preferably constitutive promoters are used, such as the 35S promoter of CaMV (Odell, Nature 313 (1985), 810-812) or promoters of the

polyubiquitin genes of maize (Christensen, Plant Mol. Biol. 18 (1982), 675-689). In order to achieve expression in specific tissues of a transgenic plant it is possible to use tissue specific promoters (see, e.g., Stockhaus, EMBO J. 8 (1989), 2245-2251). Known are also promoters which are specifically active in tubers of potatoes or in seeds of different plants species, such as maize, Vicia, wheat, barley etc. Inducible promoters may be used in order to be able to exactly control expression. An example for inducible promoters are the promoters of genes encoding heat shock proteins. Further useful promoters are described in the prior art; see, e.g.:

a) inducible promoters:
described in WO 93/21334 (=alcA/alcR system), WO 90/08826, WO 96/37609.

b) promoters active in photosynthetically active tissue:
the ST-LS1 promoter (Stockhaus et al., Proc. Natl. Acad. Sci. USA 84 (1987), 7943-7947; Stockhaus et al., EMBO J. 8 (1989), 2445-2451), the Ca/b promoter (see e.g. US-A-5,656,496; US-A-5,639,952; Bansal et al., Proc. Natl. Acad. Sci. USA 89 (1992), 3654-3658) and the Rubisco SSU promoter (see e.g. US-A-5,034,322; US-A-4,962,028) as well as the STL1 promoter (Eckes et al., Mol. Gen Genet. 205 (1986), 14-22).

c) promoters and cis-active elements mediating expression in guard cells:
- DE 19904754.5
- DE 4207358
- truncated AGPase promoter (Müller-Röber et al., Plant Cell 6 (1994), 601-612)
- Rhal promoter (Terryn et al., Plant Cell 5 (1993), 1761-1769)

d) promoters mediating expression in meristematic tissue:
- wheat histone H4 promoter (Bilgin et al., Plant Science 143 (1999), 35-44
- rice PCNA promoter (Kosugi et al., Plant J. 7 (1995), 877-886
- wheat histone H2B promoter (Yang et al., Plant Mol. Biol. 28 (1994), 155-172
- cyc07-promoter (Ito et al., Plant Mol. Biol. 24 (1994), 863-878.

Also microspore-specific regulatory elements and their uses have been described (WO96/16182). Furthermore, the chemically inducible Tet-system may be employed (Gatz, Mol. Gen. Genet. 227 (1991); 229-237). Further suitable promoters are

known to the person skilled in the art and are described, e.g., in Ward (Plant Mol. Biol. 22 (1993), 361-366). The regulatory elements may further comprise transcriptional and/or translational enhancers functional in plants cells. A plant translational enhancer often used is, e.g., the CaMV omega sequences and/or the inclusion of an intron (Intron-1 from the Shrunken gene of maize, for example) that has been shown to increase expression levels by up to 100-fold. (Maiti, Transgenic Research 6 (1997), 143-156; Ni, Plant Journal 7 (1995), 661-676). Furthermore, the regulatory elements may include transcription termination signals, such as a poly-A signal, which lead to the addition of a poly A tail to the transcript which may improve its stability. The termination signals usually employed are from the Nopaline Synthase gene or from the CaMV 35S RNA gene.

In a preferred embodiment of the recombinant DNA molecule of the invention, the subtilisin-like serine protease is derived from plants. Preferably, said plants are monocotyledonous or dicotyledonous plants such as those mentioned hereinbefore. A particular preferred embodiment of said plant is Arabidopsis.

The present invention also relates to vectors, particularly plasmids, cosmids, viruses, bacteriophages and other vectors used conventionally in genetic engineering that contain at least one recombinant DNA molecule according to the invention. Methods which are well known to those skilled in the art can be used to construct various plasmids and vectors; see, for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989). Alternatively, the recombinant DNA molecules and vectors of the invention can be reconstituted into liposomes for delivery to target cells.

Advantageously the above-described vectors of the invention comprises a selectable and/or scorable marker. Selectable marker genes useful for the selection of transformed plant cells, callus, plant tissue and plants are well known to those skilled in the art and comprise, for example, antimetabolite resistance as the basis of selection for dhfr, which confers resistance to methotrexate (Reiss, Plant Physiol. (Life Sci. Adv.) 13 (1994), 143-149); npt, which confers resistance to the aminoglycosides neomycin, kanamycin and paromycin (Herrera-Estrella,

EMBO J. 2 (1983), 987-995) and hpt, which confers resistance to hygromycin (Marsh, Gene 32 (1984), 481-485). Additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman, Proc. Natl. Acad. Sci. USA 85 (1988), 8047); mannose-6-phosphate isomerase which allows cells to utilize mannose (WO 94/20627) and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.) or deaminase from Aspergillus terreus which confers resistance to Blasticidin S (Tamura, Biosci. Biotechnol. Biochem. 59 (1995), 2336-2338). Useful scorable marker are also known to those skilled in the art and are commercially available. Advantageously, said marker is a gene encoding luciferase (Giacomin, Pl. Sci. 116 (1996), 59-72; Scikantha, J. Bact. 178 (1996), 121), green fluorescent protein (Gerdes, FEBS Lett. 389 (1996), 44-47) or ß-glucuronidase (Jefferson, EMBO J. 6 (1987), 3901-3907). This embodiment is particularly useful for simple and rapid screening of cells, tissues and organisms containing a vector of the invention. As described above, various selectable markers can be employed in accordance with the present invention. Advantageously, selectable markers may be used that are suitable for direct selection of transformed plants, for example, the phophinothricin-N-acetyltransferase gene the gene product of which detoxifies the herbicide L-phosphinothricin (glufosinate or BASTA); see, e.g., De Block, EMBO J. 6 (1987), 2513-2518 and Dröge, Planta 187 (1992), 142-151.

The present invention, also relates to host cells comprising a recombinant DNA molecule or vector of the invention. Host cells include prokaryotic and eukaryotic cells such as E. coli and yeast, respectively.

The recombinant DNA molecules according to the invention are in particular useful for the genetic manipulation of plant cells, plant tissue and plants in order to obtain plants with modified, preferably with improved or useful phenotypes as described above. Thus, the present invention relates to a method for the production of transgenic plants with altered stomata characteristica compared to wild type

plants comprising the introduction of a recombinant DNA molecule of the invention into the genome of a plant, plant cell or plant tissue.

Methods for the introduction of foreign DNA into plants as well as the selection and regeneration of transgenic plants from plant cells and plant tissue are also well known in the art. These include, for example, the transformation of plant cells, plant tissue or plants with T-DNA using Agrobacterium tumefaciens or Agrobacterium rhizogenes, the fusion of protoplasts, direct gene transfer (see, e.g., EP-A 164 575), injection, electroporation, biolistic methods like particle bombardment and other methods known in the art. The vectors used in the method of the invention may contain further functional elements, for example "left border"- and "right border"-sequences of the T-DNA of Agrobacterium which allow for stable integration into the plant genome. Furthermore, methods and vectors are known to the person skilled in the art which permit the generation of marker free transgenic plants, i.e. the selectable or scorable marker gene is lost at a certain stage of plant development or plant breeding. This can be achieved by, for example cotransformation (Lyznik, Plant Mol. Biol. 13 (1989), 151-161; Peng, Plant Mol. Biol. 27 (1995), 91-104) and/or by using systems which utilize enzymes capable of promoting homologous recombination in plants (see, e.g., WO97/08331; Bayley, Plant Mol. Biol. 18 (1992), 353-361); Lloyd, Mol. Gen. Genet. 242 (1994), 653-657; Maeser, Mol. Gen. Genet. 230 (1991), 170-176; Onouchi, Nucl. Acids Res. 19 (1991), 6373-6378). Methods for the preparation of appropriate vectors are described by, e.g., Sambrook (Molecular Cloning; A Laboratory Manual, 2nd Edition (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Suitable strains of Agrobacterium tumefaciens and vectors as well as transformation of Agrobacteria and appropriate growth and selection media are well known to those skilled in the art and are described in the prior art (GV3101 (pMK90RK), Koncz, Mol. Gen. Genet. 204 (1986), 383-396; C58C1 (pGV 3850kan), Deblaere, Nucl. Acid Res. 13 (1985), 4777; Bevan, Nucleic. Acid Res. 12(1984), 8711; Koncz, Proc. Natl. Acad. Sci. USA 86 (1989), 8467-8471; Koncz, Plant Mol. Biol. 20 (1992), 963-976; Koncz, Specialized vectors for gene tagging and expression studies. In: Plant Molecular Biology Manual Vol 2, Gelvin and Schilperoort (Eds.), Dordrecht, The Netherlands: Kluwer Academic Publ. (1994), 1-22; EP-A-120 516; Hoekema: The Binary Plant Vector System, Offsetdrukkerij

Kanters B.V., Alblasserdam (1985), Chapter V, Fraley, Crit. Rev. Plant. Sci., 4, 1-46; An, EMBO J. 4 (1985), 277-287). Although the use of Agrobacterium tumefaciens is preferred in the method of the invention, other Agrobacterium strains, such as Agrobacterium rhizogenes, may be used, for example if a phenotype conferred by said strain is desired.

Methods for the transformation using biolistic methods are well known to the person skilled in the art; see, e.g., Wan, Plant Physiol. 104 (1994), 37-48; Vasil, Bio/Technology 11 (1993), 1553-1558 and Christou (1996) Trends in Plant Science 1, 423-431. Microinjection can be performed as described in Potrykus and Spangenberg (eds.), Gene Transfer To Plants. Springer Verlag, Berlin, NY (1995).

The transformation of most dicotyledonous plants is possible with the methods described above. But also for the transformation of monocotyledonous plants several successful transformation techniques have been developed. These include the transformation using biolistic methods as, e.g., described above as well as protoplast transformation, electroporation of partially permeabilized cells, introduction of DNA using glass fibers, etc. Transgenic plant tissue and plants can be regenerated by methods well known in the art. There are various references in the relevant literature dealing specifically with the transformation of maize (cf. e.g. WO95/06128, EP 0 513 849; EP 0 465 875). In EP 292 435 a method is described by means of which fertile plants may be obtained starting from mucousless, friable granulous maize callus. In this context it was furthermore observed by Shillito et al., Bio/Technology 7 (1989), 581 that for regenerating fertile plants it is necessary to start from callus-suspension cultures from which a culture of dividing protoplasts can be produced which is capable to regenerate to plants. After an in vitro cultivation period of 7 to 8 months Shillito et al. obtain plants with viable descendants which, however, exhibited abnormalities in morphology and reproductivity.

Prioli and Söndahl, Bio/Technology 7 (1989), 589 have described how to regenerate and to obtain fertile plants from maize protoplasts of the Cateto maize inbreed Cat 100-1. The authors assume that the regeneration of protoplasts to fertile plants depends on a number of various factors such as the genotype, the physiological state of the donor-cell and the cultivation conditions. With regard to rice various transformation methods can be applied, e.g. the transformation by agrobacterium-medicated gene transfer (Hiei et al., Plant J. 6 (1994), 271-282; Hiei et al., Plant Mol.

Biol. 35 (1997), 205-218; Park et al., J. Plant Biol. 38 (1995), 365-371), protoplast transformation (Datta in "Gene transfer to plants", I. Potrykus, G. Spangenberg (Eds.), Springer-Verlag Berlin Heidelberg (1995), pages 66-75; Datta et al., Plant Mol. Biol. 20 (1992), 619-629; Sadasivam et al., Plant Cell Rep. 13 (1994), 394-396), the biolistic approach (Li et al., Plant Cell Rep. 12 (1993), 250-255; Cao et al., Plant Cell Rep. 11 (1992), 586-591; Christou, Plant Mol. Biol. (1997), 197-203) and electroporation (Xu et al., in "Gene transfer to plants", I. Potrykus, G. Spangenberg (Eds.), Springer-Verlag Berlin Heidelberg (1995), pages 201-208.

Once the introduced DNA has been integrated in the genome of the plant cell, it usually continues to be stable there and also remains with the descendants of the originally transformed cell. It usually contains a selectable marker which confers resistance against biozides or against an antibiotic such as kanamycin, G 418, bleomycin, hygromycin or phosphinotricine etc. to the transformed plant cells. The individually selected marker should therefore allow for a selection of transformed cells against cells lacking the introduced DNA.

The transformed cells grow in the usual way within the plant (see also McCormick et al., Plant Cell Rep. 5 (1986), 81-84). The resulting plants can be cultivated in the usual way and cross-bred with plants having the same transformed genetic heritage or another genetic heritage. The resulting hybrid individuals have the corresponding phenotypic properties.

Two or more generations should be grown in order to ensure whether the phenotypic feature is kept stably and whether it is transferred. Furthermore, seeds should be harvested in order to ensure that the corresponding phenotype or other properties will remain.

In general, the plants, plant cells and plant tissue which can be modified with a recombinant DNA molecule or vector according to the invention can be derived from any desired plant species. They can be monocotyledonous plants or dicotyledonous plants, preferably they belong to plant species of interest in agriculture, wood culture or horticulture, such as crop plants (e.g. maize, rice, barley, wheat, rye, oats etc.), vegetable plants such as tomato, melon, banana, chicoree, salad, cabbage or potato, tobacco, alfalfa, clover, oil producing plants (e.g. oilseed rape, sunflower, peanut, soybean, etc.), cotton, sugar beet, linseed, flax, millet, hemp, sugar cane,

leguminous plants (e.g. beans, peas etc.), wood producing plants, preferably trees, etc.

Thus, the present invention relates also to transgenic plant cells which contain a nucleic acid molecule as defined above or a recombinant DNA molecule or vector according to the invention wherein the nucleic acid molecule is foreign to the transgenic plant cell.

By "foreign" it is meant that the nucleic acid molecule is either heterologous with respect to the plant cell, this means derived from a cell or organism with a different genomic background, or is homologous with respect to the plant cell but located in a different genomic environment than the naturally occurring counterpart of said nucleic acid molecule. This means that, if the nucleic acid molecule is homologous with respect to the plant cell, it is not located in its natural location in the genome of said plant cell when stably integrated into the genome, in particular it is surrounded by different genes. In this case the nucleic acid molecule may be either under the control of its own promoter or under the control of a heterologous promoter. The nucleic acid molecule, vector or recombinant DNA molecule according to the invention which is present in the plant cell may either be integrated into the genome of the plant cell or it may be maintained in some form extrachromosomally.

In one aspect the present invention relates to a transgenic plant cell comprising stably integrated into the genome a recombinant DNA molecule of the invention or a vector of the present invention or obtainable according to the method of the invention wherein the expression of the nucleic acid molecule results in an increased expression or activity of subtilisin-like serine proteases in transgenic plants compared to wild-type plants.

The term "increase in activity" in the context of the present invention is understood to mean an increase in the expression of endogenous genes coding for a protein of the invention and/or an increase of the amount of the protein of the invention in the cells.

The increase in expression can for example be determined by measuring the amount of transcripts encoding the protein of the invention, e.g., by Northern-blot analysis, preferably by the more sensitive NASBA method (as e.g. described by

Leone et al., Journal of Virological Methods 66 (1997), 19-27; Leone et al., Nucleic Acid Res. 26 (1998), 2150-2155; Nakahara et al., Nucleic Acid Res. 26 (1998), 1854-1856) or by RT-PCR. Preferably, an increase in this context means an increase of the amount of transcripts encoding subtilases as compared to corresponding cells which are not genetically modified by at least 5%, more preferably by at least 20%, in particular by at least 50%, and most preferably by at least 400%.

Preferably, the increased expression or activity of subtilisin-like serine proteases in transgenic plants results in decreased stomata density, see, e.g., Example 7.

The increase of the amount of the protein of the invention can for example be determined by Western-blot analysis. Preferably, an increase in this context means an increase of the amount of the protein of the invention as compared to corresponding cells which are not genetically modified by at least 5%, more preferably by at least 20%, in particular by at least 50%, and most preferably by at least 400%.

Alternatively, a plant cell having a nucleic acid molecule encoding a subtilisin-like serine protease present in its genome can be used and modified such that said plant cell expresses the endogenous gene corresponding to this nucleic acid molecules under the control of heterologous promoter and/or enhancer elements. The introduction of the heterologous promoter and mentioned elements which do not naturally control the expression of a nucleic acid molecule encoding a subtilisin-like serine protease using, e.g., gene targeting vectors can be done according to standard methods, see supra and, e.g., Hayashi, Science 258 (1992), 1350-1353; Fritze and Walden, Gene activation by T-DNA tagging. In *Methods in Molecular* biology 44 (Gartland, K.M.A. and Davey, M.R., eds). Totowa: Human Press (1995), 281-294) or transposon tagging (Chandlee, Physiologia Plantarum 78 (1990), 105-115). Suitable promoters and other regulatory elements such as enhancers include those mentioned hereinbefore.

Furthermore, the present invention relates to transgenic plants or plant tissue comprising plant cells of the invention or obtainable by the above described method Preferably, the transgenic plant of the invention displays a decreased stomata density, lower conductance of stomata and/or the water consumption is lowered compared to wild type plants.

Methods for determining stomatal density, leaf conductance and water consumption comprise the following:

- Method for determining stomatal density by means of taking copies using clear nail varnish as described in Sachs et al., Annals of Botany 71 (1993), 237-243.
- Method for determining conductance as described in Muschak et al., Photosynthetica 33 (1997), 455-465.
- Methods for determining water consumption are known to the person skilled in the art.

Preferably, the transgenic plant of the invention displays one or more of the following phenotypes:

a) stomatal density: reduced by at least 2%, preferably by at least 5%, more preferably by at least 10%, most preferably by at least 30%;

b) conductance reduced by at least 2%, preferably by at least 5%, more preferably by at least 10%, most preferably by at least 25%;

c) water consumption reduced by at least 1%, preferably by at least 3%, more preferably by at least 5%, most preferably by at least 10%;

as compared to a corresponding wild type plant.

In another aspect, the present invention relates to a transgenic plant cell which contains stably integrated into the genome a recombinant DNA molecule of the invention or part thereof, a vector of the present invention or obtainable according to the method of the invention, wherein the presence, transcription and/or expression of the nucleic acid molecule or part thereof leads to reduction of the synthesis or the activity of subtilisin-like serine proteases in transgenic plants compared to wild type plants.

Usually the activity will be reduced by at least 10%, preferably by at least 30%, more preferably by at least 70%, most preferably by at least 100%. Methods of how to determine a decrease in activity as well as the definition of the term "activity" have been mentioned in the above. As it appears to be the case that, even minor changes in the amount of expression can have some effect on the phenotype of the plant methods such as NASBA analysis and RT-PCR which are considerably more sensitive in place of the Northern-blot analysis are employed for the analysis of the transgenic plant.

Preferably, said reduction is achieved by an antisense, sense, ribozyme, co-suppression in vivo mutagenesis and/or dominant mutant effect. Therefore, DNA molecules encoding an antisense RNA which is complementary to transcripts of a DNA molecule of the invention are also the subject matter of the present invention, as well as these antisense molecules. Thereby, complementarity does not signify that the encoded RNA has to be 100% complementary. A low degree of complementarity is sufficient, as long as it is high enough in order to inhibit the expression of a protein of the invention upon expression in plant cells. The transcribed RNA is preferably at least 90% and most preferably at least 95% complementary to the transcript of the nucleic acid molecule of the invention. In order to cause an antisense-effect during the transcription in plant cells such DNA molecules have a length of at least 15 bp, preferably a length of more than 100 bp and most preferably a length or more than 500 bp, however, usually less than 5000 bp, preferably shorter than 2500 bp.

The invention further relates to DNA molecules which, during expression in plant cells, lead to the synthesis of an RNA which in the plant cells due to a cosupression-effect reduces the expression of the nucleic acid molecules of the invention encoding the described protein. The invention also relates to RNA molecules encoded thereby. The principle of the cosupression as well as the production of corresponding DNA sequences is precisely described, for example, in WO90/12084, Jorgensen, Trends Biotechnol. 8 (1990), 340-344; Niebel et al. (Curr. Top. Microbiol. Immunol. 197 (1995), 91-103; Flavell et al. (Curr. Top. Microbiol. Immunol. 197 (1995), 43-36; Palaqui and Vaucheret, Plant. Mol. Biol. 29 (1995), 149-159; Vaucheret et al., Mol. Gen. Genet. 248 (1995), 311-317; de Borne et al., Mol. Gen. Genet. 243 (1994), 613-621 and in other sources.
Such DNA molecules preferably encode an RNA having a high degree of homology to transcripts of the nucleic acid molecules of the invention. It is, however, not absolutely necessary that the coding RNA is translatable into a protein.

In a further embodiment the present invention relates to DNA molecules encoding an RNA molecule with ribozyme activity which specifically cleaves transcripts of a DNA molecule of the invention as well as these encoded RNA molecules.

Ribozymes are catalytically active RNA molecules capable of cleaving RNA molecules and specific target sequences. By means of recombinant DNA techniques it is possible to alter the specificity of ribozymes. There are various classes of ribozymes. For practical applications aiming at the specific cleavage of the transcript of a certain gene, use is preferably made of representatives of two different groups of ribozymes. The first group is made up of ribozymes which belong to the group I intron ribozyme type. The second group consists of ribozymes which as a characteristic structural feature exhibit the so-called "hammerhead" motif. The specific recognition of the target RNA molecule may be modified by altering the sequences flanking this motif. By base pairing with sequences in the target molecule these sequences determine the position at which the catalytic reaction and therefore the cleavage of the target molecule takes place. Since the sequence requirements for an efficient cleavage are low, it is in principle possible to develop specific ribozymes for practically each desired RNA molecule.

In order to produce DNA molecules encoding a ribozyme which specifically cleaves transcripts of a DNA molecule of the invention, for example a DNA sequence encoding a catalytic domain of a ribozyme is bilaterally linked with DNA sequences which are homologous to sequences encoding the target protein. Sequences encoding the catalytic domain may for example be the catalytic domain of the satellite DNA of the SCMo virus (Davies et al., Virology 177 (1990), 216-224 and Steinecke et al., EMBO J. 11 (1992), 1525-1530) or that of the satellite DNA of the TobR virus (Haseloff and Gerlach, Nature 334 (1988), 585-591). The DNA sequences flanking the catalytic domain are preferably derived from the above-described DNA molecules of the invention. The expression of ribozymes in order to decrease the activity in certain proteins in cells is also known to the person skilled in the art and is, for example, described in EP-A1 0 321 201. The expression of ribozymes in plant cells was, for example, also described, in Feyter et al. (Mol. Gen. Genet. 250 (1996), 329-338).

In a preferred embodiment this reduction is effected by means of an antisense effect. For this purpose the DNA molecules of the invention or parts thereof are linked in antisense orientation with a promoter ensuring the transcription in plant cells and possibly with a termination signal ensuring the termination of the transcription as well as the polyadenylation of the transcript. In order to ensure an

efficient antisense effect in the plant cells the synthesized antisense RNA should exhibit a minimum length of 15 nucleotides, preferably of at least 100 nucleotides and most preferably of at least 500 nucleotides. Furthermore, the DNA sequence encoding the antisense RNA should be homologous with respect to the plant species to be transformed. However, DNA sequences exhibiting a high degree of homology to DNA sequences which are present in the cells in endogenic form may also be used, preferably with a homology of more than 95%. To inhibit gene expression of the nucleic acid molecule of the invention, preferably DNA molecules are used that show a homology, i.e. identity to the nucleotide sequences of SEQ ID NO: 1, 7, 9 or 11 of at least 90%, more preferably at least 93%, in particular at least 95% and most preferably at least 98%.

In a further embodiment the reduction of the amount of proteins encoded by the DNA molecules of the invention is effected by a ribozyme effect. The basic effect of ribozymes as well as the construction of DNA molecules encoding such RNA molecules have already been described above. In order to express an RNA with ribozyme activity in transgenic cells the above-described DNA molecules encoding a riboyzme are linked with DNA elements which ensure the transcription in plant cells, particularly with a promoter and a termination signal. The ribozymes synthesized in the plant cells lead to the cleavage of the mRNA encoding the subtilisin-like serine proteases described above.

Furthermore, the subtilisin-like serine protease activity in the plant cells of the invention can also be decreased by the so-called "in vivo mutagenesis" also called "chimeraplasty", for which a hybrid RNA-DNA oligonucleotide ("chimeroplast") is introduced into cells by transformation of cells (Zhu et al., Proc. Natl. Acad. Sci. 96 (1999), 8768-8773, Kipp et al., poster session at the 5th International Congress of Plant Molecular Biology, September 21-27, 1997, Singapore; Dixon and Arntzen, meeting report on "Metabolic Engineering in Transgenic Plants", Keystone Symposia, Copper Mountain, CO, USA, TIBTECH 15 (1997), 441-447; WO95/15972; Kren et al., Hepatology 25 (1997), 1462-1468; Cole-Strauss et al., Science 273 (1996), 1386-1389).

Part of the DNA component of the RNA-DNA oligonucleotide is homologous to a nucleic acid sequence of an endogenous subtilisin-like serine protease, in

comparison to the nucleic acid sequence of the endogenous subtilisin-like serine protease it displays, however, a mutation or contains a heterologous region which is surrounded by the homologous regions. By means of base pairing of the homologous regions of the RNA-DNA oligonucleotide and of the endogenous nucleic acid molecule followed by a homologous recombination the mutation contained in the DNA component of the RNA-DNA oligonucleotide or the heterologous region can be transferred to the genome of a plant cell. This results in a decrease of the activity.

In addition, the present invention relates to transgenic plants or plant tissue comprising the above described plant cells of the invention.

In a preferred embodiment the transgenic plant displays increased stomatal density, higher conductance of stomata and/or higher content of sugars and protein in plant leaves or other tissue or organs, compared to wild type plants. An increase in the stomatal density is understood to refer to an elevated content of stomata in all aerial plant organs, preferably in the leaves of plants of the present invention in the order of at least about 10% compared to the corresponding non-transformed wild type plant, which already provides for beneficial effects on the vitality of the plant such as, e.g., improved dry matter. Advantageously, the stomatal density is increased by at least about 50%, preferably by more than about 75%, particularly preferred at least about more than 100% and still more preferably more than about 200%. With respect to a decrease in the stomatal density due to the increased expression or activity of subtilisin-like serine proteases according to the invention in the transgenic plant of the invention, the stomatal density is decreased by at least 2%, preferably by more than 5%, particularly preferred at least about more than 10%, and still more preferably more than about 30%. Preferably, the transgenic plant of the invention shows a yield increase, preferably with respect to a harvestable part of the plant.
The term "yield increase" in the present context is understood to mean preferably an increase in production of ingredients, in particular soluble sugars and/or proteins and/or biomass, in particular if measured in fresh or dry weight per plant.
An increase in protein and/or sugar content in this context means that the protein content in the plant cells of the invention is increased by at least 5%, preferably by

at least 20%, in particular by at least 50% and most preferably by at least 75% as compared to plant cells of wild type plants that are not modified and/or the sugar content is increased by at least 5%, preferably by at least 25%, in particular by at least 50% and most preferably by at least 75% as compared to plant cells of wild type plants that are not modified.

Methods for determining sugar and protein content are known to the person skilled in the art.

The term "yield increase" means an increase of dry weight by least 3%, preferably by at least 10%, in particular by at least 20% and most preferably by at least 30% and/or an increase in fresh weight by least 2%, preferably by at least 5%, in particular by at least 10% and most preferably by at least 20%.

In yet another aspect, the invention also relates to harvestable parts and to propagation material of the transgenic plants according to the invention which contain transgenic plant cells described above, i.e. at least one recombinant DNA molecule or vector according to the invention and/or which are derived from the above described plants. Harvestable parts can be in principle any useful parts of a plant, for example, leaves, stems, flowers, fruit, seeds, roots etc. Propagation material includes, for example, seeds, fruits, cuttings, seedlings, tubers, rootstocks etc.

In addition, the present invention relates to a kit comprising the recombinant DNA molecule or the vector of the invention. The kit of the invention may contain further ingredients such as selection markers and components for selective media suitable for the generation of transgenic plant cells, plant tissue or plants. The kit of the invention may advantageously be used for carrying out the method of the invention and could be, inter alia, employed in a variety of applications, e.g., in the diagnostic field or as research tool. The parts of the kit of the invention can be packaged individually in vials or in combination in containers or multicontainer units. Manufacture of the kit follows preferably standard procedures which are known to the person skilled in the art. The kit or its ingredients according to the invention can be used in plant cell and plant tissue culture, for example in agriculture. The kit of the invention and its ingredients are expected to be very

useful in breeding new varieties of, for example, plants which display improved properties such as those described herein.

Thus, the present invention also relates to a method for the production of a transgenic plant comprising an increased yield and/or increased stomatal density compared to wild type plants, wherein

(a) a plant cell is genetically modified by the introduction of a foreign nucleic acid molecule the presence of which or the expression of which results in a decreased activity of a subtilase;

(b) a plant is regenerated from the cell prepared according to step (a); and

(c) further plants, if any, are generated from the plant prepared according to step (b).

Likewise, the present invention relates to a method for the production of a transgenic plant having a decreased water consumption and/or decreased stomatal density compared to wild type plants wherein

(a) a plant cell is genetically modified by the introduction of a foreign nucleic acid molecule the presence of which or the expression of which results in an increased activity of a subtilase;

(b) a plant is regenerated from the cell prepared according to step (a); and

(c) further plants, if any, are generated from the plant prepared according to step (b).

Furthermore, the present invention relates to use of at least one nucleic acid molecule encoding and/or regulating the expression of a subtilisin-like serine protease, a nucleic acid molecule hybridizing with such a nucleic acid molecule, a nucleic acid molecule encoding a product that interferes with the expression or activity of subtilisin-like serine proteases in plants, or a recombinant DNA molecule or vector of the invention in the production of transgenic plants for increasing yield, and/or increasing stomatal density, and/or increasing leaf fresh and/or dry weight, and/or increasing leaf dry matter content, and/or increasing sugar content in leaves, and/or increasing protein content in leaves, and/or increasing CO_2-assimilation, and/or sustaining photosynthesis (prevention of photoinhibition) under conditions of high irradiance (see Example 1), and/or changing the water consumption of plants, and or couteracting the consequences

of changing environmental contitions with respect to stomatal density by the inhibition or stimulation of a subtilisin-like serine protease encoding gene. Preferably such nucleic acid molecules are derived from plant genes encoding subtilases. Modulation of the activity of these genes leads to several morphological and physiological changes that are useful for the engineering of improved plants for agriculture, wood culture, or horticulture. Furthermore, the above described nucleic acid molecules and the recombinant DNA molecules and vectors according to the invention may be useful for the alteration or modification of plant/pathogene interaction. The term "pathogen" includes, for example, bacteria, viruses and fungi as well as protozoa. The plants, plant tissue and plant cells of the invention as well as harvestable parts and propagation of such plants can be used for the preparation of feed and food or additives therefor.

Deposit

One plasmid produced and used within the scope of the present invention was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures) (DSMZ) in Braunschweig, Federal Republic of Germany, which is recognized as an international depository, in accordance with the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. On October 4, 1999 the following plasmid pAH 14/58 was deposited at the German Collection of Microorganisms and Cell Cultures, (DSMZ) (Deposit number): DSM 13076

These and other embodiments are disclosed and encompassed by the description and examples of the present invention. Further literature concerning any one of the methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries, using for example electronic devices. For example the public database "Medline" may be utilized which is available on the Internet, for example under http://www.ncbi.nlm.nih.gov/PubMed/medline.html. Further databases and addresses, such as http://www.ncbi.nlm.nih.gov/, http://www.infobiogen.fr/, http://www.fmi.ch/biology/research_tools.html, http://www.tigr.org/, are known to

the person skilled in the art and can also be obtained using, e.g., http://www.lycos.com. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

The Figures show:

Figure 1: Top: Release of water vapour (transpiration) from leaves of the wildtype (wt) and the R-558 mutant (R-558) at irradiances of 300 µE (µmol m^{-2} s^{-1}) and 1200 µE (µmol m^{-2} s^{-1}) of white light measured by an infrared gas analyzer.

Bottom: Net uptake of CO_2 (assimilation) into leaves of the wildtype and the R-558 mutant (R-558) at irradiances of 300 µE (µmol m^{-2} s^{-1}) and 1200 µE (µmol m^{-2} s^{-1}) of white light measured by an infrared gas analyzer.

Figure 2: Schematic representation of the SDD1 protein marked with the amino acid positions bordering the putative pre- and the pro-sequence and the positions of the four invariant amino acids (D, H, N, S) found in all known subtilisins. Furthermore, the consequence of the mutation present in the R-558 mutant is indicated which converts the R codon at amino acid position 492 into a stop codon leading to the formation of a C-terminally truncated protein lacking the essential serine residue at position 552 (S552).

Figure 3: Schematic representation of the plasmid pG-*SDD1*

Fragment A: 7067 bp SalI – EcoRV subfragment of the BAC IGF20D22 that includes the 2328 bp coding region of *SDD1* in addition to 2 kb upstream DNA (promoter) and 2.8 kb downstream DNA was inserted into the SalI and SmaI sites of the vector pBIB-Hyg.

Vector: pBIB-Hyg (Becker, 1990, *Nucleic Acids Res.* 18, 203).

Figure 4: Schematic representation of the plasmid p35S-*SDD1*

Fragment A: 35S promoter of the Cauliflower Mosaic Virus; (Gardner et al., 1981, *Nucleic Acids Res.* 9, 2871-2888)

Fragment B: 2328 nucleotides coding region of the SDD1 gene (SEQ ID No. 1)

Fragment C: polyadenylation signal of the gene 3 from the T-DNA of the Ti-plasmid pTi ACH 5 (Gielen et al., 1984, *EMBO J.* 3, 835-846)

Vector: pBIB-Hyg (Becker, 1990, *Nucleic Acids Res.* 18, 203).

Figure 5: Sequence alignments in the four highly conserved domains, the D region, the H region, the substrate binding site, and the S region of the subtilisins Subtilisin BPN´ (Wells et al. 1983, Nucleic Acids Res. 11, 7911-7925), the KEX2 of yeast (Mizuno et al. 1988, *Biochem. Biophys. Res. Commun.* 156, 246-254), the human FURIN/PACE (Wise et al. 1990, *Proc. Natl. Acad. Sci USA* 87, 9378 - 9382), the human PC1/PC3 (Seidah et al., 1991, *Mol. Endocrinol.* 5, 111 - 122; Smeekens et al., 1991, *Proc. Natl. Acad. Sci. USA* 88, 340 - 344), the CUCUMISIN from *Cucumis melo* (Yamagata et al., 1994, *J. Biol. Chem.* 269, 32725 - 32731), LeP69 from *Lycopersicon esculentum* (Tornero et al., 1996, *Proc. Natl. Acad. Sci. USA* 93, 6332 - 6337), the AG12 from *Alnus glutinosa* (Ribeiro et al., 1995, *Plant Cell* 7, 785 - 794) and of SDD1. The positions of the invariant amino acids are marked with *. Identical amino acids present at corresponding positions in the different subtilisins are highlighted with black boxes.

Figure 6: Schematic representation of the plasmid p35S-α*SDD1*

Fragment A: 35S promoter of the Cauliflower Mosaic Virus; (Gardner et al., 1981, *Nucleic Acids Res.* 9, 2871-2888)

Fragment B: 2079 bp – fragment (position 74 – 2153 according to the sequence shown in SEQ ID No. 1) of the *SDD1* gene inserted in antisense orientation to the 35S promoter.

Fragment C: polyadenylation signal of the gene 3 from the T-DNA of the Ti-plasmid pTi ACH 5 (Gielen et al., 1984, *EMBO J.* 3, 835-846)

Vector: pBIB-Hyg (Becker, 1990, *Nucleic Acids Res.* 18, 203).

Figure 7: Amino acid comparison of three different subtilases; at_subp1=Subtilase from Arabidopsis thaliana, see SEQ ID No. 2, st_subp1=Subtilase from Solanum tuberosum, see SEQ ID No. 8; st_subp2=Subtilase from Solanum tuberosum, see SEQ ID No. 12.

The Examples illustrate the invention:

Example 1: H_2O transpiration and CO_2 assimilation are increased in the *Arabidopsis thaliana* R-558 mutant particularly under conditions of high irradiance

Arabidopsis thaliana R-558 mutant plants and corresponding wildtype plants (wt) were grown until bolting in soil (Einheitserde Typ P / Einheitserde Typ T / sand: 2 / 1 / 1) under standard culture conditions in a climatised growth chamber at 16 h photoperiod (180 µmol m^{-2} s^{-1} fluorescent light; lamp type: TLD36W/840 and TLD36W/830, Philips, Hamburg, Germany) with day and night temperature and relative humidity of 20°C, 60% relative humidity and 16°C, 75% relative humidity, respectively. Single leaves (n=10) were clamped into a gas exchange measurement chamber of an infrared gas analyzer (Walz, Effeltrich, Germany) with H_2O release from as well as CO_2 uptake into the leaves were measured according to the procedure described by Muschak et al. (*Photosynthetica* 33, 455-465, 1997). As shown in Figure 1, the leaves of the mutant plants showed increased transpiration of H_2O and increased assimilation (net uptake) of CO_2 under low light (300 µmol m^{-2} s^{-1}) and under high light (1200 µmol m^{-2} s^{-1})

conditions applied during the measurements. Enhancement of CO_2 assimilation, being almost double in the R-558 mutant in comparison to the wild type, was most prominent under the high light conditions which caused a depression of CO_2 assimilation in the wild type in comparison to the low light conditions (photoinhibition).

Example 2: Isolation of the *SDD1* gene through map-based gene cloning

The genetic locus affected by the mutation in the R-558 mutant has previously been mapped to the top arm of the *Arabidopsis thaliana* chromosome 1 to an interval of approximately 0.59 cM bordered by the molecular markers IGF-20G19LE and IGF-25I3RE (D. Berger, 1997, PhD Thesis Freie Universität Berlin). Two clones of the *Arabidopsis thaliana* genomic IGF-BAC library (Mozo et al., 1998, *Mol. Gen. Genet.* 258, 562-570), IGF20D22 and IGF21M11, which fully cover this region, were sequenced by the SPP consortium (see http://sequence-www.stanford.edu/ara/SPP.html) as part of the *Arabidopsis* genome initiative (Bevan et al., 1997; *Plant Cell* 9, 476-478; http://genome-www.stanford.edu/Arabidopsis/agi.html). The 0.59 cM region was thus identified to cover 113 kb of genomic DNA sequence. In order to identify the *SDD1* gene corresponding to the mutant locus, this region was scanned for mutations by application of the restriction SSCP- (single strand conformational polymorphism) technique (Dean and Gerrard, 1991, *BioTechniques* 10, 332 - 333; Iwahana et al., 1992, *BioTechniques* 12, 64 - 66) which for this purpose was adapted for the use in plants. This approach is novel and has not been applied for mutation scanning in plants before. Thus, 57 DNA fragments of 2-kb each, were separately PCR amplified from total DNA of wild type and R-558 plants and after digestion with Alu I and/or Hinf I they were analysed through polyacrylamid gel electrophoresis as described by Dean and Gerrard, 1991 (*BioTechniques* 10, 332 – 333) and Iwahana et al., 1992 (*BioTechniques* 12, 64 – 66). A single SSCP was detected that discriminated between the two genotypes and which upon sequencing of the corresponding DNA fragments was shown to be caused by a single C/G -> T/A mutation (Seq. ID No. 1; Seq. ID No. 3). This mutation introduced a premature stop codon into an ORF of a predicted gene spanning 2328 bp that encoded for a

deduced polypeptide of 775 amino acids (Fig. 2; Seq. ID No. 2; Seq. ID No. 4; Genbank Accession AC002411; http://pgec-genome.pw.usda.gov/F20D22.anno.html#anchor12).

Example 3: Genetic complementation of the R-558 mutant by *Agrobacterium tumefaciens* – mediated DNA-transfer

In order to confirm the identity of the 2328 bp DNA sequence (Seq. ID No. 1;) as the protein coding region of the *SDD1* gene defective in the R-558 mutant, genetic complementation experiments were performed with the introduction of a wild type DNA-copy into the R-558 mutant through *Agrobacterium tumefaciens* – mediated genetic transformation.

Two plasmids were generated for this purpose:

Plasmid pG-*SDD1'* (Fig. 3) carries the 7067 bp SalI - EcoRV subfragment of the BAC IGF20D22 that includes the 2328 bp coding region of *SDD1* in addition to 2 kb upstream DNA (promoter) and 2.8 kb downstream DNA was inserted into the SalI and SmaI sites of the T-DNA vector pBIB-Hyg (Becker, 1990, *Nucleic Acids Res.* 18, 203).

The second plasmid, p35S-*SDD1* (Fig. 4), harbours the three fragments, A, B, C, inserted into the pBIB-Hyg vector (Becker, 1990, *Nucleic Acids Res.* 18, 203). Fragment A, which was inserted between the EcoRI and SacI restricition sites in the polylinker of pBIB-Hyg, includes the 35S promoter of the Cauliflower Mosaic Virus (CaMV) comprising the nucleotides 7146 through 7464 as described by Gardner et al. (*Nucleic Acids Res.* 9, 2871-2888, 1981). Fragment C contains the polyadenylation signal of the gene 3 from the T-DNA of the Ti-plasmid pTi ACH 5 (Gielen et al., *EMBO J.* 3, 835 – 846, 1984), nucleotides 11749 through 11939 which was isolated as Pvu II - Hind III fragment from the plasmid pAGV 40 (Herrera-Estrella et al., *Nature* 303, 209 – 213, 1983) and which, after addition of a Sph I linker to the Pvu II restriction site, was inserted into the Sph I and Hind III restriction sites of pBIB-Hyg. The resulting intervening plasmid was called pBIN-AR-Hyg. Fragment B covers the 2328 nucleotides coding region of the *SDD1* gene (Seq. ID No. 1) that was amplified by PCR from the BAC IGF20D22 and

provided with Asp718 and XbaI linker sequences and which was inserted into the Asp718 and XbaI restriction sites of pBIN-AR-Hyg.

Both plasmids were separately introduced into *Agrobacterium tumefaciens* according to the procedure described by Höfgen and Willmitzer (*Nucleic Acids Res.* 16, 9877, 1988) and the corresponding T-DNAs were stably introduced into the R-558 mutant by *Agrobacterium tumefaciens* - *in planta* transformation following the method described by Bechtold et al. (*Compt. Rend. Acad. Sci.* 316, 1194 – 1199, 1993). Transformed seedlings selected for antibiotic (Hygromycin) resistance were grown to maturity and tested for the expression of mutant or wildtype phenotypes by microscopic examination of rosette leaves cleased with 80 % ethanol.

Table 1: Analysis of stomatal density and distribution in the abaxial epidermices of cotyledons and leaves of wild type (wt), mutant (R-558) and transgenic mutant (R-558 / G-SDD1; R-558 /35S-SDD1)

Plant	Cotyledon			Primary Leaf			
	Single Stomata[a]	Clustered Stomata[b]	n[c]	Single Stomata[a]	Clustered Stomata[b]	n[c]	Density [no./mm²]
wt #1	100 %	0 %	58	100 %	0 %	163	97.0
wt #2	100 %	0 %	40	100 %	0 %	174	124.3
wt #3	100 %	0 %	51	98.9 %	1.1 %	176	125.7
wt #4	100 %	0 %	49	100 %	0 %	160	114.3
wt #5	100 %	0 %	58	100 %	0 %	166	118.6
R-558 #1	61 %	39 %	136	86 %	14 %	492	351.4
R-558 #2	56 %	44 %	62	90.1 %	9.9 %	421	300.7
R-558 #3	54 %	46 %	137	89.6 %	10.4 %	395	282.1
R-558 #4	58 %	42 %	109	93.9 %	6.1 %	409	292.1
R-558 #5	60 %	40 %	85	91.6 %	8.4 %	403	287.9
R-558 / G-*SDD1* #1	100 %	0 %	47	100 %	0 %	279	199.3
R-558 / G-*SDD1* #2	100 %	0 %	53	83.4 %	16.6 %	181	161.6
R-558 / G-*SDD1* #3	100 %	0 %	52	100 %	0 %	139	99.3
R-558 / G-*SDD1* #4	96.4 %	3.6 %	55	100 %	0 %	195	139.3
R-558 / G-*SDD1* #5	100 %	0 %	53	100 %	0 %	180	128.6
R-558 / G-*SDD1* #6	100 %	0 %	55	100 %	0 %	169	120.7
R-558 / G-*SDD1* #7	100 %	0 %	53	100 %	0 %	163	116.4
R-558 / G-*SDD1* #8	96.8 %	3.2 %	62	100 %	0 %	285	203.6
R-558 / G-*SDD1* #9	100 %	0 %	37	100 %	0 %	98	70.0
R-558 / G-*SDD1* #10	96.7 %	3.3 %	60	100 %	0 %	199	142.1
R-558 / 35S-*SDD1* #1	82 %	18 %	66	100 %	0 %	167	119.3
R-558 / 35S-*SDD1* #2	100 %	0 %	34	100 %	0 %	123	87.9
R-558 / 35S-*SDD1* #3	93 %	7 %	55	100 %	0 %	168	120
R-558 / 35S-*SDD1* #4	74 %	26 %	80	100 %	0 %	136	97.1
R-558 / 35S-*SDD1* #5	100 %	0 %	45	100 %	0 %	122	87.1
R-558 / 35S-*SDD1* #6	80 %	20 %	70	100 %	0 %	195	139.3
R-558 / 35S-*SDD1* #7	53 %	47 %	131	98 %	2 %	200	142.9
R-558 / 35S-*SDD1* #8	91 %	9 %	68	100 %	0 %	123	87.9
R-558 / 35S-*SDD1* #9	80 %	20 %	90	95.1 %	4.9 %	123	87.9
R-558 / 35S-*SDD1* #10	94 %	6 %	65	100 %	0 %	108	77.1

[a] Stomata separated from other stomata by at least one epidermal cell. [b] Stomata placed in direct contact to at least one other stoma. [c] Number of stomata sampled.

As shown in Table 1, 7 out of 10 and 2 out of 10 transformants harbouring the T-DNAs of the pG-*SDD1* or the p35S-*SDD1* plasmids, respectively, showed a wildtype phenotype on cotyledons with respect to the appearance/absence of clustered stomata. 7 transformants carrying the T-DNA of p35S-*SDD1* showed an intermediate phenotype in cotyledons due to inappropriate expression of the transgene in this organ. In primary leaves, all 10 transformants harboring the T-DNA of pG-*SDD1* and all 10 transformants carrying the T-DNA of p35S-*SDD1* showed a strong reduction in stomatal density and/or the fraction of clustered stomata as compared to the R-558 mutant. These data unequivocally demonstrated the identity of the 2325 bp DNA fragment as the coding region of the *SDD1* gene.

Example 4: Analysis of the *SDD1* nucleotide sequence and SDD1 amino acid sequence

The analysis of the *SDD1* nucleotide and derived amino acid sequences was performed using the GCG 8.1 and BLAST 2.0 computer programs (see: Program Manual for the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711; Altschul et al. 1997, *Nucleic Acids Res.* 25, 3389-3402).

The derived amino acid sequence of *SDD1* shows significant identity / similarity to known members of a specific class of serine proteases, called subtilisins or dibasic processing endoproteases. In subtilisins, four regions form the catalytic triad and the substrate binding site and are most highly conserved among subtilisins, prominent representatives of which are the bacterial SUBTILISIN BPN' (Wells et al. 1983, Nucleic Acids Res. 11, 7911-7925), the KEX2 of yeast (Mizuno et al. 1988, *Biochem. Biophys. Res. Commun.* 156, 246-254), the human furin/PACE (GenBank, Acc. No. X17094) and PC1/PC3 (Seidah et al., 1991, *Mol. Endocrinol.* 5, 111 - 122; Smeekens et al., 1991, *Proc. Natl. Acad. Sci. USA* 88, 340 - 344). Several genes from plants encoding subtilases have been isolated such as CUCUMISIN from *Cucumis melo* (Yamagata et al., 1994, *J. Biol. Chem.* 269, 32725 - 32731), P69 from *Lycopersicon esculentum* (Tornero et al., 1996, *Proc. Natl. Acad. Sci. USA* 93, 6332 - 6337), or AG12 from *Alnus glutinosa*

(Ribeiro et al., 1995, *Plant Cell* 7, 785 - 794). The *in vivo* functions of these plant enzymes, however, are hitherto unknown. In the four conserved regions, SDD1 displays highest sequence similarity to the subtilisins listed above and contains the four characteristic invariant amino acids present in all subtilisins hitherto known (Fig. 5). This unequivocally proves the belonging of SDD1 to this class of endoproteases. The amino acid sequence motif VICAAGNNG within the substrate binding site, however, is unique and distinguishes *SDD1* from all other known subtilisins. The mutation present in the R-558 mutant creates a premature stop codon leading to the formation of a C-terminally truncated protein which lacks the essential S-domain containing the catalytically active serine residue (Fig. 2).

Example 5: Modulation of stomatal density in plants through modulation of *SDD1* expression by genetic engineering

The usefulness of the *SDD1* gene for the creation of plants with various different levels of increased or decreased stomatal densities through modulation of the degree of *SDD1* gene expression was shown by the analysis of further transgenic plants. 4 out of 10 of the transgenic plants carrying the aforementioned (Example 4) T-DNA of p35S-*SDD1* and 1 out of 10 of the transgenic plants carrying the aforementioned (Example 4) T-DNA of pG-*SDD1* showed lower stomatal density than the corresponding wildtype plants analyzed in parallel (Table 1).

Furthermore, *SDD1* antisense inhibition studies were performed: To this end, the plasmid p35S-α*SDD1* was generated which contains an antisense-gene construct called `35S-α*SDD1*´ (Fig. 6). A 2079 bp - fragment (position 74 - 2153 according to the sequence shown in seq. ID 1) of the *SDD1* gene was PCR-amplified and subcloned into the pCR 2.1 - vector (Invitrogen, Leek The Netherlands). Using the flanking Asp718 (3´) and XbaI (5´) restriction sites, the 2 kb *SDD1* - fragment was cut from the pCR 2.1 - vector and inserted into the Asp718 and XbaI sites of the pBIN-AR-Hyg. vector (see example 3), thus placing it in antisense orientation to the CaMV 35S – promoter.

The plasmid p35S-α*SDD1* was introduced into *Agrobacterium tumefaciens* according to Höfgen and Willmitzer (*Nucleic Acids Res.* 16, 9887, 1988) and was used to generate transgenic *Arabidopsis thaliana* plants through application of the

procedure described by Schmidt and Willmitzer (*Plant Cell Rep.* 7, 583-586, 1988). Among the transgenic plants carrying the T-DNA of p35S-α*SDD1* thus generated, individuals with increased stomatal density were obtained.

It was thus demonstrated that through the application of genetic engineering techniques, a gene encoding a subtilisin-like serine protease, can be used to generate plants with various different levels of decreased or increased stomatal densities brought about by the modulation of the expression of said gene.

Example 6: Cloning of two SDD1 homologs from *Solanum tuberosum*

A 2328 bp fragment representing the complete SDD1 coding region from *Arabidopsis* was amplified from the clone IGF20D22 of the *Arabidopsis thaliana* genomic IGF-BAC library (Mozo et al., 1998, Mol. Gen. Genet. 258, 562-570) via PCR and was used as a radiolabeled probe (Random Primed DNA Labeling Kit, Boehringer Mannheim) in the screening procedure.

Plaque lifting was performed on 1.6 x 10^6 pfus of a genomic library from *Solanum tuberosum* L. line AM80/5793 (Liu et al., 1991, Plant Mol. Biol. 17, 1139-1154), using Hybond N filters (Amersham).

After pre-hybridization for 4 h at 42^0 C in buffer A (5 x SSC, 0,5 % BSA, 5 x Denhardt, 1 % SDS, 40 mM phosphate buffer, pH 7.2, 100 mg/l herring sperm DNA, 25 % formamid) filters were hybridized to the radiolabeled probe (see above). After 8 h of hybridization the filters were washed 3 times for 20 min at 50^0C in a buffer containing 3 x SSC and 0.5 % SDS. X-ray film exposure was usually performed for 14h.

Ten strongly hybridizing phage plaques were rescreened and purified to homogenity. Phage DNA was prepared according to the method described by Patterson & Dean 1987 (NAR Vol.15 (15), 6298).

According to their restriction patterns and Southern analysis, using the radiolabeled *SDD1* PCR fragment as a probe, phages were divided into two different classes. In case of class I a 3864 bp hybridizing EcoRI/SalI fragment was subcloned into pMCS5 (EcoRI/SalI) (Mo Bi Tec, Göttingen), yielding plasmid pAH10/58. In case of class II a ~4.5 kb hybridizing ScaI- fragment was subcloned in pMCS5 (Mo Bi Tec, Göttingen), yielding plasmid pAH 14/58. Both of the plasmids were subjected to DNA-sequencing analysis and contained the

nucleotide sequences, coding for *SDD1* homologs from *Solanum tuberosum* P1gen (SEQ ID NO: 7), P1 - corresponding to P1gen without the intron - (SEQ ID NO: 9), and P2 (SEQ ID NO: 11) The class I a fragments are characterized by the presence of introns, class IIa fragments by the absence of introns. The amino acid sequences encoded by P1 and P2 are shown in SEQ ID NOS: 8, 10 and 12, respectively, and compared to that of SDD1 in Figure 7.

Example 7: Overexpression of subtilase in tobacco

A genomic fragment of 2.3 kb was amplified by PCR from the genome of A. thaliana var. C24, and Asp 718/ Xba I (the primers comprised these sequences) and cloned into the vector pBinAR Hyg (Höfgen and Willmitzer (1990), Plant Sci. 66: 221-230).

Agrobacteria GV2260 (Deblaere (1985), Nucl. Acid Res. 13: 4777-4788) were transformed by way of heat shock, and tobacco plants were infected cv. SNN and regenerated (Rosahl et al., (1987) EMBO J. 6: 1155-1159).

In total, 24 plants were examined as to their RNA expression. For this, RNA from leaf plants was prepared according to Logemann et al. (1987) Anal. Biochem. 163: 16-20) in tissue culture (22°C, 50% atmospheric humidity, 2000 Lux, 16h/8h light rhythm). About 10µg RNA were loaded onto a denaturating gel (Lehrach et al., (1977) Biochem. 16: 4743-4751) and thereupon transferred onto a positively charged membrane Hybond N+ (Amersham Buchler, Braunschweig) via capillary transfer. Thereafter, the RNA was fixed onto the membrane via heat fixation (2h/80°C), washed in 2XSSC for a short period of time (2-3 min), prehybridized for at least 1h at 65°C (0.25M Na-P buffer pH 7.2; 1% BSA, 7% SDS and 10mM EDTA) and hybridized overnight at 65°C with a radioactively labeled probe (Feinberg, and Vogelstein, (1983) Anal. Biochem. 132: 6-13). After two washes with 2XSSC at 65°C for 30 min each the filter was exposed on an X-ray filter overnight at –80°C.

In total, 24 plants were analyzed by way of "Northern technique", of which 12 could be classified as positive, 9 being classified as strong and 3 as weaker expressers. Copies using clear nail varnish were prepared from these plants as well as from the wild type, 5 leafs from different plants of the wild type were analyzed. For doing so, 5 areas each were counted and stomatal density was

determined as stomata/mm^2. When doing so, it was surprisingly found that stomatal density on the adaxial leaf surface was about twice as high as on the abaxial surface; see table 2.

Table 2

Microscopic analysis of stomatal density

WT/adaxial

Pl.-Nr.						Stomata/mm2		
1	71	65	73	68	69	56		
2	58	69	62	51	52	47		
3	78	74	68	73	76	60		
4	83	87	71	82	73	64		
5	77	75	79	76	73	62		
6	69	69	64	68	63	54		
7	61	67	61	64	63	51		
					MV:	**56**	STD:	6,13

WT/abaxial

Pl.-Nr.						Stomata/mm2		
1	101	108	105	98	109	84		
2	128	144	140	139	147	113		
3	163	154	160	157	165	129		
4	111	115	116	121	107	92		
5	183	176	180	168	171	142		
6	131	138	135	141	139	111		
					MV:	**112**	STD:	21,79

AR

Subt./adaxial

Pl.Nr.						Stomata/mm2		
13	27	21	20	23	21	18		
14	17	23	24	22	20	17		
15	21	20	23	19	22	17		
16	17	13	19	18	23	15		
19	24	28	29	26	28	22		
22	25	23	27	22	26	20		
30	22	26	24	21	25	19		
37	27	26	30	33	28	23		
38	30	26	33	30	28	24		
40	27	23	26	25	31	21		
41	21	23	25	22	20	18		
42	27	25	22	23	22	19		
					MV:	**19**	STD:	2,72

AR

Subt./abaxial

Pl.Nr.						Stomata/mm2
13	74	71	72	71	76	59
14	73	61	72	70	76	57
15	80	78	71	83	75	63
16	86	99	72	83	97	71
19	84	95	87	93	94	73

22	58	47	62	62	56	46
30	93	93	96	87	91	75
37	92	97	95	100	93	77
38	76	80	78	77	83	64
40	81	77	88	73	76	64
41	62	56	61	68	59	50
42	82	75	78	81	75	63
mean value					MV:	63

STD: 9,58

	WT	AR Subt.
adaxial	56	19
abaxial	112	63

STD = standard deviation
MV = mean value
WT = wild type
AR = transgenic plant

After microscopic analysis of the copies of the transgenic plants which were taken by using clear nail varnish, a decrease in stomatal density by about 50% on both leaf sides could be detected. The hypostomatic phenotype, however, is also found in the transgenic plants; see table 2.

No differences could be found between strong and weak "expressers"; thus even a minor increase of SDD1 activity seems to be sufficient to result in a phenotype.